Biochemical Pharmacology, Vol. 36, No. 13, pp. 2203–2208, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

ALTERED PROTEIN PHOSPHORYLATION IN INTACT RAT CORTICAL SYNAPTOSOMES AFTER *IN VIVO* ADMINISTRATION OF FLUPHENAZINE

PHILLIP J. ROBINSON*† and PETER R. DUNKLEY‡

†Merrell Dow Research Institute, Cincinnati, OH 45215, U.S.A.; and ‡The Neuroscience Group, Faculty of Medicine, University of Newcastle, N.S.W., Australia, 2308

(*Received* 29 *August* 1986; *accepted* 17 *December* 1986)

Abstract—Phenothiazines such as fluphenazine are able to inhibit calcium-stimulated protein kinases *in vitro* in both lysed and intact synaptosomes. In this study protein phosphorylation was assayed in intact synaptosomes isolated from the cerebral cortex of rats treated chronically (21 days, 10 mg/kg, i.p.) or acutely (1 hr, 10 mg/kg, i.p.) with fluphenazine. When intact synaptosomes from chronically treated animals were prelabeled with $^{32}P_i$, there were two effects on protein phosphorylation: an increase in the basal labeling of many phosphoproteins and a decrease in depolarization-evoked protein phosphorylation. Acute injections had even more pronounced effects, but the direction and nature of the effects were the same. No effects on K^+-stimulated calcium entry or on protein phosphatase activity were detected. When lysed synaptosomes from chronically treated animals were labeled in the presence of [γ-^{32}P]ATP, a small decrease in calmodulin-dependent and cAMP-dependent protein phosphorylation was observed. The results suggest that two different *in vivo* mechanisms may underlie these effects, and these are discussed. We proposed that intact synaptosomes may be a good model in which to study the *in vivo* mechanisms of the action of fluphenazine since they appear to retain at least some effects of the drug after subcellular fractionation.

Neuroleptic drugs such as phenothiazines are capable of altering neuronal protein phosphorylation *in vitro* by inhibiting calmodulin [1] and hence calmodulin-dependent protein kinases [2–4] and by inhibiting protein kinase C [5]. Thus, fluphenazine inhibits calcium-dependent protein phosphorylation *in vitro*, both in lysed synaptosomes labeled with [γ-^{32}P]ATP [2] and in intact synaptosomes labeled with $^{32}P_i$ [3, 6, 7]. Similar effects have also been observed in other intact cells [8] where the same mechanisms may apply.

Several studies on the effects of *in vivo* neuroleptic drug administration on neuronal protein phosphorylation have been undertaken (for a review see Ref. 9). Administration of the neuroleptic haloperidol was found to have two effects. Chronic treatment increased basal phosphorylation when synaptic membranes from treated rats were "post hoc" phosphorylated with [γ-^{32}P]ATP [10], correlating with partial phosphorylation and activation of tyrosine hydroxylase [11, 12]. The mechanism is not understood, but it may involve increased cAMP-dependent protein phosphorylation (e.g. Ref. 13). The second effect is decreased calcium-stimulated phosphorylation, probably due to calmodulin inhibition, since the effect is overcome by the addition of exogenous calmodulin [10]. The difficulty with these types of studies is preservation of the *in vivo* state of phosphorylation of substrate proteins during subcellular fractionation, and the nonphysiological conditions used for the phosphorylation assays.

The aim of this study was to determine whether the previously described *in vitro* effects of fluphenazine on protein phosphorylation in intact synaptosomes [7] could also be observed after *in vivo* drug administration. The approach employed was to isolate intact synaptosomes from drug-treated rats and examine depolarization-stimulated protein phosphorylation after prelabeling with $^{32}P_i$. The advantage of this approach over "post hoc" procedures is that an intact, functional fraction of nerve endings is isolated which retains the relative activities of protein kinases and protein phosphatase and that phosphorylation occurs under conditions more relevant to the *in vivo* situation. Similar procedures have been used to show altered protein phosphorylation in synaptosomes from opiate-treated animals [14, 15]. In control experiments we also investigated calcium uptake and cAMP- and calmodulin-stimulated "post hoc" phosphorylation in order to establish the nature of the *in vivo* effects of fluphenazine.

METHODS

Drug administration. Adult male Wistar rats weighing approximately 300 g were employed throughout this study. Paired rats from the same litter were injected intraperitoneally with fluphenazine in a vehicle of 0.9% NaCl adjusted to pH 5 with minimum HCl (treated), or with vehicle alone (control). Acutely-injected animals received a single injection of 10 mg/kg, whereas chronically-

* Address correspondence to: Dr. Phillip J. Robinson, Merrell Dow Research Institute, Merrell Dow Pharmaceuticals Inc., 2110 East Galbraith Road, P.O. Box 156300, Cincinnati, OH 45215.

injected animals received 10 mg/kg daily at 9:00 a.m. for 21 days. The initial tranquilizing effect of fluphenazine injection was apparent within 15 min and was present for up to 90 min. Rats treated chronically showed considerable tolerance to the drug within 7 days, and from that day were tranquilized for only very short periods (approximately 20 min). After an initial loss of body weight during the first 5 days of chronic injections, fluphenazine had no effect on the rate of subsequent weight gain over the next 2 weeks. Rats were killed by stunning and decapitation 1 hr after the last injection.

Protein phosphorylation. A crude synaptosomal fraction (P2 fraction*) was isolated from whole cerebral cortex [16] and maintained intact by resuspension in a modified Krebs buffer usually containing 1.2 mM calcium [16] or lysed by resuspension and homogenization in 30 mM Tris, pH 7.4 [2]. Protein concentrations [17] were finally adjusted to 5 mg/ml for intact synaptosomes and 10 mg/ml for lysed synaptosomes. Intact synaptosomes were prelabeled for 45 min in the presence of $^{32}P_i$ followed by brief incubations in control or depolarizing (41 mM K^+ final concentration) buffer, as previously described [16, 18]. Lysed synaptosomes were phosphorylated for 30 sec with [γ-^{32}P]ATP as previously described [2, 19] in the presence or absence of EGTA (1.0 mM), cAMP (50 μM), calcium (1.1 mM) or calcium plus calmodulin (12.5 μg/ml). All reactions were terminated by addition of SDS stop buffer, and the samples were subjected to polyacrylamide gel electrophoresis on gradient gels of 7.5–15% acrylamide and autoradiographed as previously described [16]. Autoradiographs were scanned and total protein phosphorylation was obtained by integration of the total area under the scan, whereas individual phosphoproteins were quantitated by measuring peak heights in comparison with controls always run on the same gel. Exposures were selected such that only the linear response range of the film was employed [7, 16, 18–20]. The results obtained by densitometry were compared with results obtained after excision of individual phosphoprotein bands and counting by liquid scintillation or by acid precipitation of whole protein and counting. These procedures produced qualitatively the same results as densitometry. Phosphoproteins are referred to by their apparent molecular mass in kilodaltons using the terminology previously described [16, 18, 21]. The specific phosphoproteins referred to in Results were identified by a number of criteria [20, 21] as being identical to the previously described phosphoproteins synapsin Ib (phosphoprotein 75), protein IIIa and IIIb (phosphoproteins 72 and 59) and B-50 (phosphoprotein 45). Variability between controls with intact synaptosomes was assessed in separate experiments by comparing control levels of ^{32}P incorporation in synaptosomes from different animals on the same gel. For total protein phosphorylation, where one lane was set at 100%, variation was ±3.5 (N = 5 experiments performed in duplicate). Variation for individual proteins was virtually the same (e.g. P91, ±3.9; P75, ±3.4; P59, ±6.3; and P45, ±4.5).

Calcium uptake. Calcium uptake was determined as previously described [7].

* Abbreviations: P2 fraction, postmitochondrial pellet; SDS, sodium dodecyl sulfate; and EGTA, ethyleneglycolbis (amino-ethylether)tetra-acetate.

RESULTS

Control phosphorylation in intact synaptosomes. *In vivo* fluphenazine injections produced significant increases in the control, or basal, level of protein phosphorylation in crude synaptosomes prelabeled in the presence of 1.2 mM calcium (Table 1). Total protein phosphorylation was increased significantly by 25% in chronically injected rats and this increase was even greater (37%) in acutely injected animals. These increases were observed with most of the individual phosphoproteins (Fig. 1A, compare lanes 1 and 6). The extent of the effect of chronic injections was similar for individual phosphoproteins 91, 75, 72, 59, and 45 (Table 2). These phosphoproteins were chosen as they had been extensively inves-

Table 1. Effect of *in vivo* fluphenazine administration on total protein phosphorylation in intact synaptosomes

	Total protein phosphorylation (%)				
	1.2 mM Calcium			0.1 mM Calcium	
Condition	Sham	Chronic	Acute	Sham	Acute
Control	100 ± 2.0	125 ± 7.8*	137 ± 9.6*	100 ± 1.1	95 ± 1.6
Depolarized	131 ± 2.8	143 ± 10.0	152 ± 9.6	124 ± 1.8	104 ± 2.9
Difference	31	18†	15†	24	9†

Rats were injected i.p. either chronically or acutely with 10 mg/kg fluphenazine, and a cortical P2 fraction was isolated. The P2 fraction was prelabeled with $^{32}P_i$ for 45 min in the presence of 1.2 mM calcium (or 0.1 mM for low calcium), and aliquots were incubated for 5 sec in control or 41 mM K^+ buffer (depolarized). Results are expressed relative to controls from sham animals, run on the same gel. Data are means ± SEM from three experiments performed in duplicate.

* Significant differences from sham control, $P < 0.01$ (Student's *t*-test).

† Significant difference from sham depolarized, $P < 0.01$ (Student's *t*-test).

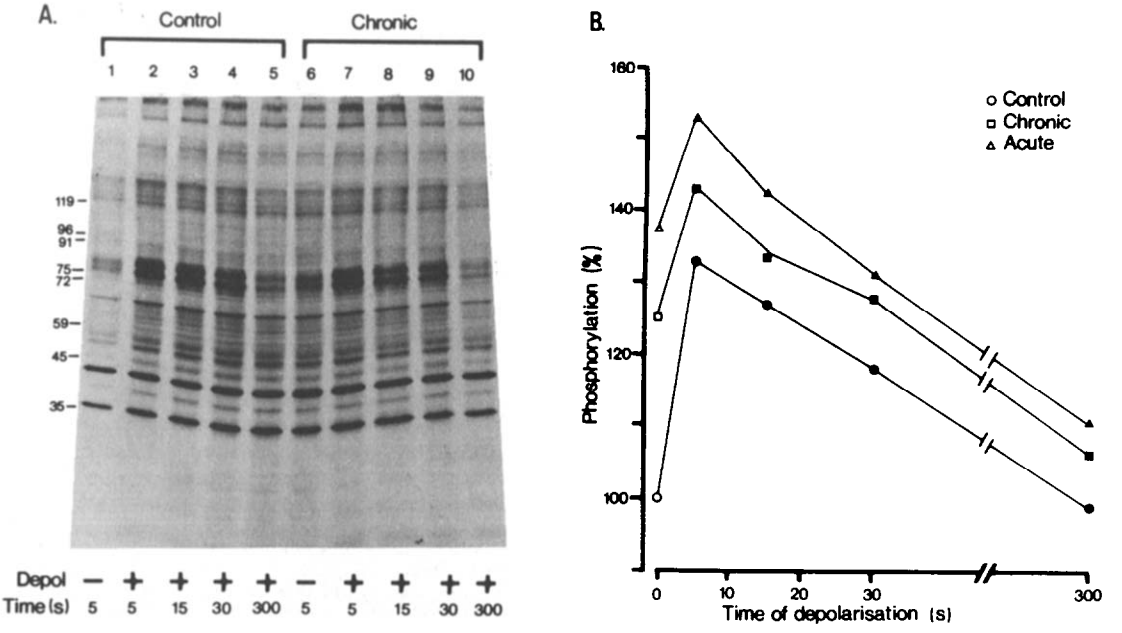

Fig. 1. Effect of chronic or acute injections of fluphenazine on intact synaptosomal protein phosphorylation. Rats were given chronic or acute (21 days) injections of fluphenazine (10 mg/kg, i.p.) and killed 1 hr after the last injection. A P2 fraction was prepared and prelabeled with $^{32}P_i$ for 45 min in the presence of 1.2 mM calcium, followed by incubation for 5 sec in control buffer (A, −; B, open symbols) or for various times in depolarizing buffer (41 mM K^+; A, +; B, closed symbols). (A) Autoradiograph of phosphoproteins from control and chronically-injected animals. (B) Quantitation of the effect on total protein phosphorylation of sham (circles), chronic (squares), or acute (triangles) injections. Results are means from six (control), three (chronic) or four (acute) experiments, each performed in duplicate, and the largest SEMs were 4.1, 11.8 and 10.5 respectively. Variability for control values is indicated in the Methods section.

tigated previously [20, 21], and they represented proteins phosphorylated by cyclic AMP-dependent (91 and 59), calcium/calmodulin-dependent (75 and 72), and calcium/phosphatidylserine-dependent (45) protein kinases. When synaptosomes from acutely injected animals were prelabeled and incubated with 0.1 mM calcium, the increase in control phosphorylation produced by fluphenazine was abolished (Table 1).

Depolarization-stimulated phosphorylation. Fluphenazine injections significantly reduced the stimulation of protein phosphorylation due to depolarization (Table 1). Chronic injections produced an approximately 50% reduction in depolarization-stimulated protein phosphorylation, and similar results were found after acute treatment. The decrease was seen for most of the individual phosphoproteins whose labeling was normally increased on depolarization (Fig. 1A, Table 2). Depolarization-stimulated protein phosphorylation was also reduced relative to controls when the level of calcium present during the prelabeling and incubation was lowered to 0.1 mM after acute injections (Table 1). When depolarization of the synaptosomes

Table 2. Effect of *in vivo* chronic fluphenazine administration on the phosphorylation of individual proteins

Rat	Condition	Phosphorylation (%)				
		Phosphoprotein				
		91	75	72	59	45
Sham	Control	100	100	100	100	100
	Depolarized	123 ± 2.4	180 ± 4.2	150 ± 3.5	132 ± 1.9	137 ± 4.2
	Difference	23	80	50	32	37
Fluphenazine (chronic)	Control	116 ± 9.4	123 ± 7.5	123 ± 9.7	113 ± 6.2	117 ± 8.6
	Depolarized	127 ± 9.8	194 ± 14.3	164 ± 9.6	139 ± 7.8	146 ± 11.5
	Difference	11	71	41	26	29

Experimental details are as described in the legend to Table 1, and protein phosphorylation is expressed relative to sham controls run on the same gels. Data are means ± SEM for three treatment groups and three separate preparations performed in duplicate. The identity of certain individual phosphoproteins has been determined previously [20, 21]. P75 is synapsin Ib, P72 is protein IIIa, P59 is protein IIIb, and P45 is the B-50 protein. Variability for control values is indicated in the Methods section.

Table 3. Effect of chronic fluphenazine on synaptosomal calcium uptake

Rat	Depolarized	Calcium uptake nmol/mg protein	Calcium uptake Percent
Sham	–	1.68 ± 0.13	100
Sham	+	4.10 ± 0.19	244
Chronic	–	1.56 ± 0.39	93
Chronic	+	4.32 ± 0.66	257

Rats were injected chronically with 10 mg/kg fluphenazine daily for 21 days, and a P2 fraction was prepared. This fraction was preincubated at 37° for 45 min in the presence of 1.2 mM calcium before determination of calcium uptake over a 5-sec incubation in either control or depolarizing buffer. SEMs are indicated for three experiments, each performed in triplicate.

is extended to 300 sec, dephosphorylation of synaptosomal proteins occurs [16, 18] due to phosphatase activity. The rates of dephosphorylation for synaptosomes from the control, chronic and acute fluphenazine conditions were the same (Fig. 1B).

Calcium uptake. Chronic injections of fluphenazine did not alter either control or depolarization-stimulated calcium uptake (Table 3).

Phosphorylation in lysed synaptosomes. Total protein kinase activity in lysed synaptosomes was reduced by approximately 13% for cyclic AMP- and calmodulin-stimulated protein kinases after chronic administration of fluphenazine (Fig. 2). No drug-related effect was seen with calcium alone or in the presence of EGTA, and control levels of protein phosphorylation were unaltered. The efffect of fluphenazine on total protein kinase activity was not reflected in any individual phosphoprotein (Table 4).

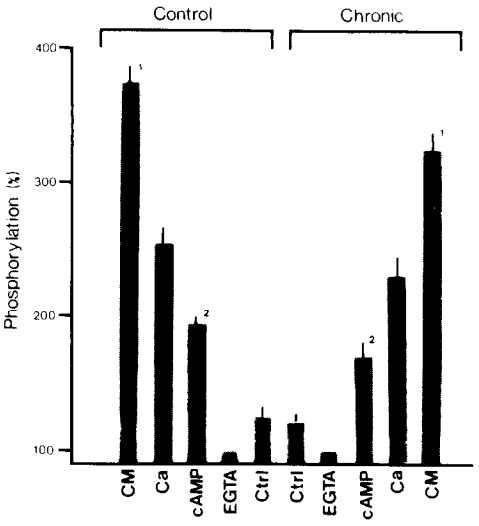

Fig. 2. Effect of chronic fluphenazine injections on the [γ-^{32}]ATP labeling of lysed synaptosomes. After chronic fluphenazine, the P2 fraction was lysed and labeled for 30 sec with [γ-^{32}P]ATP in the presence of no addition (ctrl), EGTA (1.0 mM), cAMP (50 μM, plus EGTA), calcium (1.1 mM, plus EGTA), or calmodulin (CM 12.5 μg/ml, plus calcium and EGTA). Results show total protein phosphorylation and are from three experiments performed in duplicate. SEMs are indicated. Key: significant difference from each other; $^{(1)}P < 0.02$; and $^{(2)}P < 0.05$.

DISCUSSION

In a previous study from our laboratory, it was shown that addition of fluphenazine to intact synaptosomes (from untreated animals) produces two major effects [7]: first, an increase in basal protein phosphorylation at lower drug concentrations and, second, an inhibition of depolarization-dependent activation of calcium-stimulated protein kinases at higher drug concentrations due to an inhibition of calcium/phosphatidylserine- and calcium/calmodulin-dependent protein kinases. The major finding of the present study is that the same two major effects of fluphenazine can be observed in synaptosomes

Table 4. Effect of chronic fluphenazine administration on the phosphorylation of individual phosphoproteins in lysed synaptosomes

Condition	Chronic fluphenazine	Phosphorylation (%) Phosphoprotein 75	59	63	48
cAMP	–	314 ± 13	236 ± 10.3		
	+	297 ± 22 (95%)	229 ± 10.1 (97%)		
Ca^{2+}/CM	–	372 ± 26		622 ± 22	623 ± 18
	+	341 ± 29 (92%)		547 ± 45 (88%)	580 ± 47 (93%)

Rats were injected chronically with 10 mg/kg fluphenazine, and a lysed P2 fraction was labeled in the presence of [γ-^{32}P]ATP. The phosphorylation of individual phosphoproteins in the presence of cAMP or calcium/calmodulin is shown, relative to a control containing EGTA which was set at 100%. The identity of these proteins has been determined previously [20, 21]; P75 is synapsin Ib, P59 is protein IIIb, and P63 and P48 are subunits of calmodulin-dependent protein kinase II. Values in parentheses are the percent of chronic fluphenazine phosphorylation relative to control. Results are from three experiments performed in duplicate; SEMs are indicated. There was no statistically significant differences between any pairs. Variability for control values is indicated in the Methods section.

isolated from drug-treated animals. It is proposed that the same mechanisms operating after *in vitro* drug addition may be occurring *in vivo*. The possible mechanisms underlying the alterations in protein phosphorylation include: effects on protein kinases (possibly via effects on cyclic nucleotides, calcium and/or calmodulin), protein phosphatases, or on substrate availability.

The first effect of *in vivo* fluphenazine was that it increased the basal levels of protein phosphorylation in intact synaptosomes. Currently, the underlying mechanism of this effect is unknown. It is clear, however, that the fluphenazine effect is broadly based, modifying the phosphorylation of a number of proteins approximately equally. From the substrates affected this suggests that the effects may be due to alterations in the relative activities of at least cyclic AMP- and calcium/calmodulin-dependent protein kinases. However, since these protein kinases are active in non-stimulated synaptosomes [20], a decreased basal protein phosphatase activity could also underlie the effect. Increased basal phosphorylation was observed at 1.2 mM calcium, but not at 0.1 mM. In a previous report, we have shown that basal phosphorylation can also be regulated by the concentration of calcium present during the prelabeling and incubation [18]. Thus, decreasing calcium from 1.2 mM to 0.1 mM also increased basal phosphorylation. The abolition of the effect of fluphenazine on basal phosphorylation at 0.1 mM calcium may, therefore, be due to a maximal basal phosphorylation already having been achieved by lowered calcium alone and suggests that fluphenazine may modulate resting intracellular calcium levels. This finding may be used to separate the two components of fluphenazine's actions on protein phosphorylation and allow the inhibition of depolarization-induced protein phosphorylation to be examined alone. In other studies, neuroleptic-induced increases in dopaminergic activity [22] led to increased phosphorylation and activation of tyrosine hydroxylase [11, 12]. Cortical synaptosomes were employed in our study, and the increased basal phosphorylation is, therefore, not specifically associated with the dopaminergic system.

The "post hoc" phosphorylation experiments support the view that increased basal activities of cyclic AMP- and calcium/calmodulin-dependent protein kinases may have occurred *in vivo*, as well as in isolated intact synaptosomes. Thus, we found a decreased "post hoc" activity of these two protein kinases in synaptosomes isolated from chronically treated rats. Since the incubation times employed yielded maximum phosphorylation levels, a decrease in "post hoc" phosphorylation reflects an increased *in vivo* phosphorylation which led to fewer phosphorylation sites being available on the substrate proteins for the *in vitro* labeling [23]. The data suggest that protein kinase activities are regulated by fluphenazine rather than a specific effect on a single major synaptosomal phosphoprotein. In other studies, chronic treatment with non-phenothiazine neuroleptics has been associated with alterations in calmodulin localization [24] and "post hoc" protein phosphorylation [10, 24–26] in striatal membranes. However, the different classes of drugs employed in these studies may not relate to the effects of fluphenazine.

The second effect of *in vivo* fluphenazine administration was a reduction in depolarization-dependent protein phosphorylation. The immediate explanation, that this apparent inhibition is due simply to the prior increase in basal labeling (see Fig. 1), cannot fully account for the effect since inhibition was still observed when the basal effect was abolished by the use of low calcium buffers. The effect was shown not to result from an alteration in depolarization-stimulated calcium uptake, correlating with previous work [27], or in protein phosphatase activity, since no alterations in the rate or extent of dephosphorylation were observed in this study. The simplest explanation of this finding is that *in vivo* fluphenazine administration produces an inhibition of the extent to which calcium-stimulated protein kinases can be activated, as also observed upon fluphenazine addition to the *in vitro* assay [7].

The results of this study raise the possibility that the *the vivo* mechanisms of action of fluphenazine can be studied in isolated intact synaptosomes from drug-treated animals. The advantages of the intact synaptosome model are that they retain at least some of the effects of the drug administration throughout their isolation, that some of these effects are mimicked by addition of exogenous fluphenazine, and that they have the potential to be utilized to study possible effects of drugs on other factors such as neuronal calcium levels [28, 29] or protein kinase and calmodulin translocations [15]. Synaptosomes are, therefore, a potential model for some of the possible *in vivo* mechanisms of action of other psychotropic drugs and disorders of synaptic transmission.

REFERENCES

1. B. Weiss, W. C. Prozialeck and T. L. Wallace, *Biochem. Pharmac.* **31**, 2217 (1982).
2. P. R. Dunkley and P. J. Robinson, *Biochem. J.* **199**, 269 (1981).
3. R. J. DeLorenzo, *Cell Calcium* **2**, 365 (1981).
4. J. C. Juskevich, D. M. Kuhn and W. Lovenberg, *J. biol. Chem.* **258**, 1950 (1983).
5. B. C. Wise and J. F. Kuo, *Biochem. Pharmac.* **32**, 1259 (1983).
6. R. J. DeLorenzo, *Fedn Proc.* **41**, 2265 (1982).
7. P. J. Robinson, P. E. Jarvie and P. R. Dunkley, *J. Neurochem.* **43**, 659 (1984).
8. R. L. Kenigsberg, A. Cote and J. M. Trifaro, *Neuroscience* **7**, 2277 (1982).
9. J. E. Wilson, in *Biochemistry of Brain* (Ed. S. Kumar), p. 523. Pergamon Press, Oxford (1980).
10. Y. S. Lau and M. E. Gnegy, *Life Sci.* **30**, 21 (1982).
11. C. Bakhit and J. Gibb, *Life Sci.* **25**, 1389 (1979).
12. M. A. Lazar, I. N. Mefford and J. D. Barchas, *Biochem. Pharmac.* **31**, 2599 (1982).
13. Y. H. Ehrlich and E. G. Brunngraber, *Trans. Am. Soc. Neurochem.* **7**, 109 (1976).
14. J. C. Juskevich, J. P. O'Callaghan and W. Lovenberg, *Fedn Proc.* **38**, 253 (1979).
15. D. H. Clouet and N. Williams, *Neurochem. Res.* **7**, 1135 (1982).
16. P. J. Robinson and P. R. Dunkley, *J. Neurochem.* **41**, 909 (1983).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).

18. P. J. Robinson and P. R. Dunkley, *J. Neurochem.* **44**, 338 (1985).
19. P. R. Dunkley and P. J. Robinson, *Neurochem. Res.* **8**, 865 (1983).
20. P. R. Dunkley, C. M. Baker and P. J. Robinson, *J. Neurochem.* **46**, 1692 (1986).
21. P. R. Dunkley and P. J. Robinson, *Prog. Brain Res.* **69**, 273 (1986).
22. N. M. J. Rupniak, P. Jenner and C. D. Marsden, *Life Sci.* **32**, 2289 (1983).
23. E. J. Nestler and P. Greengard, *Protein Phosphorylation in the Nervous System*. John Wiley, New York (1984).
24. M. E. Gnegy, *Fedn Proc.* **41**, 2273 (1982).
25. N. Williams and D. H. Clouet, *J. Pharmac. exp. Ther.* **220**, 278 (1982).
26. Y-S. Lau, C. Runice, F. J. Dowd and C. Anene, *Proc. west. Pharmac. Soc.* **26**, 139 (1983).
27. S. W. Leslie, S. C. Elrod, R. Coleman and J. K. Belknap, *Biochem. Pharmac.* **28**, 1437 (1979).
28. H. Yamamoto, R. A. Harris, H. H. Loh and E. L. Way, *J. Pharm. exp. Ther.* **205**, 255 (1978).
29. D. B. Chapman and E. L. Way, *A. Rev. Pharmac. Toxic.* **20**, 553 (1980).